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Attachment Stimulates Exopolysaccharide Synthesis by a Bacterium

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This study examined the hypothesis that solid surfaces may stimulate attached bacteria to produce exopolymers. Addition of sand to shake-flask cultures seemed to induce exopolymer synthesis by a number of subsurface isolates, as revealed by optical microscopy. Several additional lines of evidence indicated that exopolymer production by attached cells (in continuous-flow sand-packed columns) was greater than by their free-living counterparts. Total carbohydrates and extracellular polysaccharides, both normalized to cell protein, were greater (2.5- and 5-fold, respectively) for attached cells than for free-living cells. Also, adsorption of a polyanion-binding dye to the exopolymer fraction was sixfold greater for attached cells than for unattached cells. When surface-grown cells were resuspended in fresh medium, exopolymer production decreased to the level characteristic of unattached cells, which ruled out the possibility that attached cells comprised a subpopulation of sticky mucoid variants. The mechanism by which attachment stimulated exopolymer synthesis did not involve changes of the specific growth rate, growth stage, or limiting nutrient.

Bacteria have devised complex regulatory circuits controlling exopolymer synthesis at the level of gene expression (6, 22). The commonly reported association of exopolymers with bacteria living attached to solid surfaces raises the question of whether these regulatory circuits controlling exopolymer synthesis respond to the proximity of a solid surface. This possibility was implied by a model of time-dependent active adhesion (16) which proposed that following an initial stage of weak adhesion, certain bacteria become firmly attached through the synthesis of anchoring exopolymers. Despite the importance of the sessile mode of growth in natural, industrial, or medical systems, it remains unclear whether a solid surface does in fact stimulate exopolymer production.

Numerous microscopic analyses have shown that bacteria attached to solid surfaces in natural environments are often embedded in exopolymers (see, e.g., reference 7). These observations, however, do not demonstrate an effect of solid surfaces on exopolymer synthesis, because various alternate mechanisms could lead to the same observations. For example, the species composition of the attached community may differ from that of the free-living cells or the solid surface may prevent exopolymers from diffusing into the surrounding medium. The latter mechanism was suggested by Wrangstadh et al. (28), who found that the presence of a solid surface accelerated the appearance of cell-bound exopolymers during the initial stages of starvation of *Pseudomonas* sp. strain S9. Additionally, apparent surface effects on exopolymer production may in fact reflect the effect of specific growth rate (27) which may be different at the solid-liquid interface and in the bulk liquid (see, e.g., reference 26).

Bengtsson (4) observed that a subsurface bacterial isolate attached to gravel in a continuous-flow microcosm was associated with greater amounts of exopolymer than the cells collected with the effluent stream. Again, such observations cannot be taken as proof for surface-induced exopolymer synthesis because, as pointed out by Silverman et al. (21), preferential attachment of a subpopulation of mucoid variants might explain the data as well. Such subpopulations differing in the level of exopolymer production are frequently observed within single clones (2, 23).

The putative effect of solid surfaces on exopolymer synthesis may be an indirect one if the altered medium composition at the phase boundary changes the nutritional status of the attached bacteria (reviewed in reference 26), which in turn may affect exopolymer synthesis (24). Alternatively, exopolymer synthesis by attached cells may be responding directly to external signals, such as contact with a surface, by means of membrane-embedded signal-transducing proteins which are important components of the regulatory circuits controlling exopolymer synthesis in several bacteria (6). The actual external signals sensed by these membrane sensors remain unknown. External signals have been identified for the bacterium *Pseudomonas aeruginosa*, but the sensory proteins have not been discovered yet. This organism regulates transcription of exopolymer genes in response to medium osmolarity (5, 23) or dehydrating agents (10). Recently, Davies et al. (9) showed that activation of a gene (*algC*) for the exopolymer alginate was higher for *P. aeruginosa* attached to a Teflon mesh than for the unattached bacteria.

In the present study, we examined the synthesis of exopolymers by various subsurface bacterial isolates growing attached to clean sand in continuous-flow columns. We propose that cell attachment can stimulate exopolymer production independently of changes in the growth stage, growth rate, or type of limiting nutrient.

MATERIALS AND METHODS

Bacterial strains and media. The strains were obtained from the Department of Energy Subsurface Microbial Culture Collection, routinely cultured in PYG medium (proteose peptone, 5 g; yeast extract, 10 g; glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; in 1 liter of deionized water) and maintained at −70°C in glycerol. They had originally been isolated in the laboratory of David Balkwill (Florida State University) from aquifer samples taken at

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depths ranging from 145 to 259 m at the Savannah River Site drilling site P24, South Carolina. Strains CAP and SLI are subclones (25) of gram-negative Subsurface Microbial Culture Collection isolate B0693. All strains produce mucoid colonies on PYG plates, are nonmotile, and do not ferment glucose (Api Rapid NFT test; Analytab Products, Plainview, N.Y.).

Except where noted (see Results), all experiments were performed with a 33-fold dilution of medium C. Medium C (pH 6.8), which was C limiting (see Discussion), contained (in grams per liter): trisodium citrate dihydrate (1), NH_4Cl (0.17), $MgSO_4 \cdot 7H_2O$ (0.7), $CaCl_2 \cdot 2H_2O$ (0.07), $Na_2HPO_4 \cdot 7H_2O$ (1.6), KH_2PO_4 (0.78), yeast extract (0.018), Casamino Acids (0.018), EDTA (0.004), $FeCl_3$ (0.004), and trace elements solution (0.25 ml) (18).

Screening strains in batch cultures. Each strain was grown in 20-ml glass vials with 0.8 ml of medium C and 2 g of unwashed silica sand (40 to 100 mesh; Fisher Scientific Co., Pittsburgh, Pa.) with shaking. After 4 days, the sand was gently rinsed with deionized water and vortexed at maximum speed. Adherent cells extracted by the vortex-wash steps were negatively stained with India ink (12) and examined by light microscopy for the presence of extracellular polymers. Cells were compared with control cultures incubated without sand.

Carbohydrate synthesis at various growth rates. Fed-batch cultures were used to examine the effect of growth rate on total carbohydrate synthesis. Exponentially growing cells in 2, 80, or 180 ml of medium C were fed with fresh medium C at a constant rate of 1.4 ml/h with shaking (fed-batch cultures). After 17 h, we measured total carbohydrate, protein, and cell abundance. Growth rates were estimated from the dilution rate at the time of sampling. Identical measurements were performed on log-phase batch cultures (maximal growth rate, 0.46 h^{-1}).

Continuous-flow apparatus. Attached bacteria were grown in Pasteur pipets filled with 3 g of sand (see description above) and fed with a 33-fold dilution of medium C at a constant flow rate of 1.4 ml/h (upward flow). The sand had been washed in concentrated HCl, thoroughly rinsed in deionized water, sonicated, and combusted at 550°C. The entire flow apparatus was autoclaved before inoculation. The pipets were inoculated 2 h before the flow was started with approximately 0.5 ml of an overnight culture in undiluted medium C.

Prior to measuring bacterial abundance and various chemical properties, attached cells were recovered by transferring the sand to a culture tube, draining the excess liquid with a small pipet to remove unattached cells, and vortexing the sand repeatedly (20 s; 10 times) at maximum speed with 1-ml aliquots of deionized water.

Growth rates estimated from [^{3}H]thymidine incorporation. Approximately 0.1 g of wet sand was transferred immediately after the flow was stopped to 1 ml of fresh flow medium with 0.05 nmol of [^{3}H]thymidine (72.5 Ci/mmol; NEN Research Products, Boston, Mass.). The exact weight of dry sand was measured after radioassaying. After a 30-min incubation with shaking, [^{3}H]thymidine incorporation was halted by cooling the sample on ice and by addition of 0.5 nmol of unlabelled thymidine. After 15 min, the macromolecules were precipitated by adding 100 μl of ice-cold 50% trichloroacetic acid, except for the killed controls to which trichloroacetic acid had been added before the incubation. The precipitates together with the sand particles were collected on 0.45-μm-pore-size Millipore HA filters and washed twice with ice-cold 10% trichloroacetic acid and once with ice-cold 70% ethanol. The filters were air dried and radioassayed with a Beckman liquid scintillation counter. The assay was calibrated with log-phase batch cultures in medium C. The conversion factor was 3.1×10^{18} cells per mol of thymidine for the CAP strain. Specific growth rates were calculated by dividing bacterial production by cell abundance measured simultaneously on separate subsamples. Growth rates of attached bacteria may be underestimated by this approach because diffusion may limit the availability of [^{3}H]thymidine. This error was probably small, however, because cell density was very low, implying little difference in the availability of dissolved compounds for assimilation by attached and unattached cells.

Bacterial abundance. All cell counts were determined by epifluorescence microscopy of acridine orange-stained cells. When testing was performed on attached cells recovered from the sand (see the description above), cell clumps were disrupted by briefly (30 s) sonicating the sand extract.

Alcian blue adsorption assay for exopolymers. A semiquantitative assay was developed for cell-bound exopolymers, analogous to the ruthenium red adsorption method for measurement of extracellular polysaccharides in sludge flocs (13). Cells recovered from the sand were centrifuged and resuspended in 2 ml of phosphate buffer (12 mM; pH 6.8). After removal of a 1-ml aliquot for protein assay, 8 μl of a 1% alcian blue 8GX (Sigma Chemical Co., St. Louis, Mo.) aqueous solution was added. After 5 min, the cells were pelleted and the adsorbance of the supernatant measured at 606 nm. This adsorbance was compared to that of a control without cells. The amount of cell-bound dye was then the decrease in dye adsorbance in the supernatant after cells (with dye bound to exopolymers) were removed by centrifugation. There was no detectable dye adsorption in extracts from uninoculated pipets after 1 week of continuous flow.

In certain cases, adsorption to the exopolymer fraction was determined by measuring the difference between adsorption to whole cells and adsorption to cells from which the bulk of the capsular polymers had been removed by brief sonication (see the assay description below).

Carbohydrate and protein assays. Carbohydrates were assayed by the phenol-sulfuric acid method (11). For the extracellular polysaccharide assay, 1-ml cell suspensions were placed in 1.8-ml plastic centrifuge tubes and sonicated for 20 s at 26 W (setting 3.5) with a Cell Disruptor 200 (Branson Sonic Power Co., Danbury, Conn.). This treatment effectively stripped the bacteria of most of their capsular material without causing a significant amount of cell lysis (<10%), as shown by cell counts and negative staining of capsules. The carbohydrate assay was carried out with the supernatant after pelleting the cells. The pellet was used for the protein assay. When the exopolysaccharide assay was performed on unattached cells growing in flow medium, the supernatant was concentrated with a stirred ultrafiltration cell (Amicon, Danvers, Mass.) equipped with a YM3 membrane (molecular weight cut-off, 3,000).

Cell proteins were solubilized in 0.5 N NaOH at 60°C for 20 min and assayed colorimetrically by the method of Bradford (Bio-Rad Laboratories, Richmond, Calif.). Standards were prepared with bovine serum albumin in 0.1 N NaOH.

Light microscopy. The cells were gently heat fixed, stained with a 2% crystal violet–0.8% ammonium oxalate solution in 19% ethanol, and examined with an Olympus BH-2 light microscope.

TABLE 1. Effect of sand on exopolymer synthesis by various subsurface bacteria in batch cultures

Strain	Polymer synthesis	
	No sand	With sand
B0428	−[a]	+++
B0577	−	++
B0483	−	+
B0693 CAP	+	+
B0693 SLI	−	−
B0550	−	−

[a] −, no exopolymer; +, small (< 0.5 μm) capsule; ++, medium (0.5 to 1.0 μm) capsule; +++, capsule and slime layers.

RESULTS

Screening of various strains for sand-induced exopolymer synthesis. The effect of sand addition on exopolymer production by different subsurface isolates was examined in batch cultures. Though all strains produced exopolymers in PYG medium, exopolymers were not detected, as revealed by negative staining results with India ink, in medium C without the addition of sand (except for the CAP strain, which synthesized a small capsule). When sand was added, however, exopolymers were associated with attached cells of several strains (Table 1).

Effect of growth rate and attachment on total cellular carbohydrate content. Surface effect on exopolymer synthesis was further examined in continuous-flow sand-packed columns in order to increase the yield of the attached population and at the same time decrease the background of unattached cells. Only the results obtained with the CAP strain are reported here, because exopolymer synthesis by this strain did not differ significantly at various growth rates or stages (see results below). The specific growth rates of cells attached to the sand, measured by [^{3}H]thymidine incorporation, were 0.10 ± 0.06 h^{-1} and 0.05 ± 0.01 h^{-1} at the inlet end and outlet end of the columns, respectively. These rates are much lower than that for unattached cells growing exponentially in shake flasks on flow medium (0.40 h^{-1}). At the time of the measurement (after 3.5 days of flow), the attached bacteria covered less than 1% of the solid-liquid interfaces; the attached cell densities were 1.0 ± 0.2 and 0.6 ± 0.2 × 10^8 cells per g of sand at the inlet end and outlet end, respectively.

Because the specific growth rates of attached cells and unattached control cultures differed, we first examined the effect of growth rate on exopolymer production. In this first set of experiments, exopolymer production was estimated by measuring total cell carbohydrates. In fed-batch cultures of unattached cells growing on undiluted medium C, a 100-fold decrease in specific growth rate resulted in a 3-fold decrease in total carbohydrate per cell (Fig. 1A). This decrease in carbohydrate content was proportional to the decrease in the amount of protein per cell, resulting in a total carbohydrate/protein ratio that changed little with specific growth rate and oscillated around 2.0 μg of carbohydrate per μg of protein (Fig. 1B). For attached cells, the ratios were 4.4 ± 1.0 and 5.8 ± 1.7 μg of carbohydrate per μg of protein in the inlet end and outlet end of the columns, respectively (Fig. 1B). These data indicate that at equal specific growth rates the total carbohydrate content of attached cells was about 2.5-fold greater than that for unattached cells.

This difference between attached and unattached cells may have resulted from the use of a more diluted nutrient

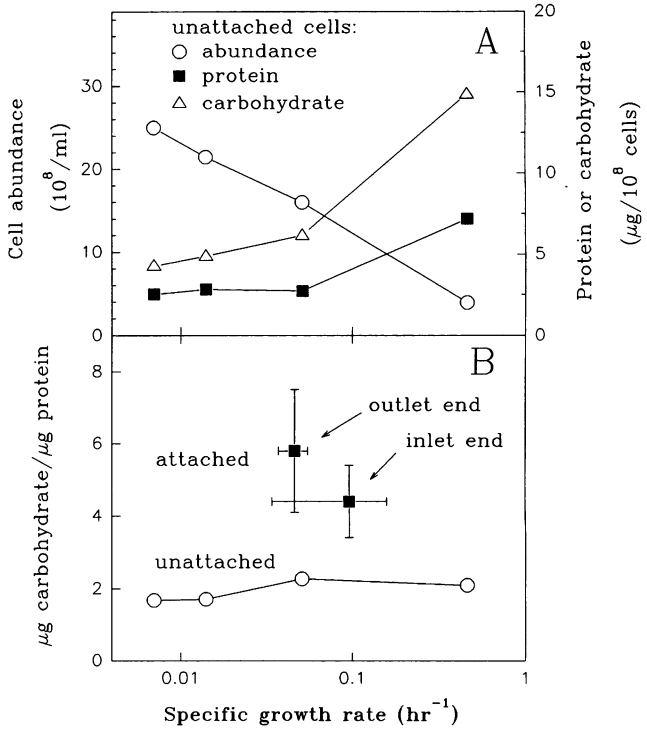

FIG. 1. (A) Effect of growth rate on cell protein and cell carbohydrate for CAP strain growing unattached in medium C. (B) Effect of attachment on cell carbohydrates. Values for attached cells represent the means of three independent measurements (± standard deviations [error bars]).

medium in the flow-through columns than in the fed-batch cultures rather than from attachment per se. However, this was unlikely because the exopolysaccharide/protein ratio for unattached cells did not vary significantly when medium C was diluted (data not shown). Diluted medium C was used in all following experiments.

Effect of attachment on exopolysaccharide production. The larger carbohydrate/protein ratio of attached cells did not indicate whether the surplus carbohydrate was stored intracellularly or deposited outside the cells as exopolymers. To address this question, we separated extracellular material from the cells and compared the carbohydrate content of these extracellular fractions (exopolysaccharides) from attached and unattached cells. Attached cells had an average exopolysaccharide/protein ratio of 4.6 ± 0.9 (Fig. 2A), whereas effluent cells resuspended in fresh flow medium (without shaking) maintained a ratio close to 1.0 throughout the growth cycle, although it decreased slightly during the stationary phase (Fig. 2B). A value of 1.0 indicated that cells of the CAP strain produced a small capsule in the absence of cell attachment. This was confirmed by microscopic analysis. The exopolysaccharide/protein ratio for attached cells decreased slightly as the density of cells attached to the sand grains increased threefold (Fig. 2A), but this decrease is not statistically significant.

The cells recovered from the sand columns typically formed very large clumps comprising 50 to 500 cells even at attached cell densities lower than 2 × 10^8 cells per g of sand. The cells within these clumps were interconnected by amorphous material that stained with crystal violet (Fig. 3A). On the other hand, the cells collected in the effluent stream were

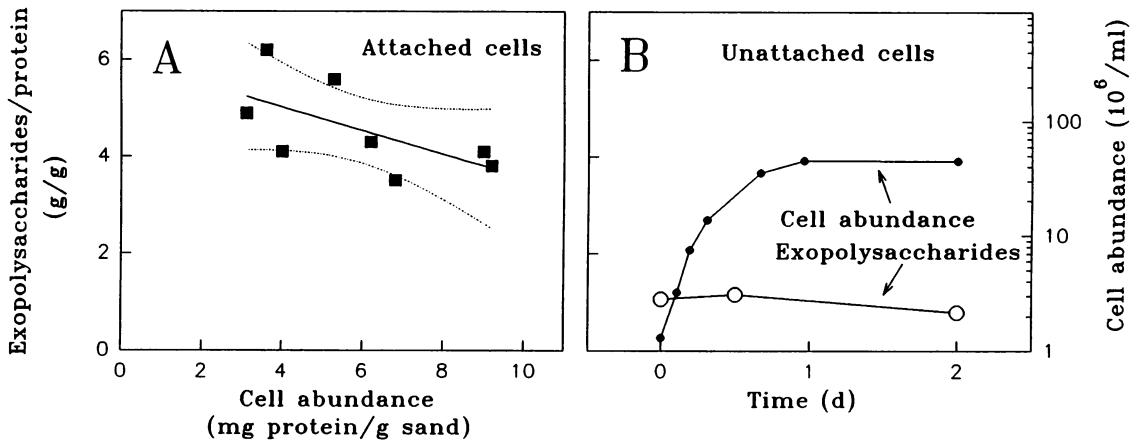

FIG. 2. (A) Exopolysaccharide of attached cells recovered from the sand columns. (B) Unattached cells collected in the effluent and diluted with fresh flow medium. The straight line is the linear regression and the dashed lines delineate the 95% confidence interval. d, day.

single and devoid of crystal violet-stained extracellular material (Fig. 3B).

Effect of detachment on exopolymer production. We developed a semiquantitative assay for cell-bound exopolymers based on the adsorption of alcian blue, a dye known to bind polyanions (14). This assay, which estimates the amount of exopolymer by the decrease in absorbance of the supernatant following exposure to cells, is very similar to the ruthenium red adsorption method employed by Figueroa and Silverstein (13) to measure extracellular polysaccharide in sludge flocs. We used alcian blue because it stained extracellular material but not cells, as revealed by light microscopy. The relationship between the amount of adsorbed dye and the abundance of encapsulated cells was highly linear (Fig. 4). However, after most of the capsular material had been removed by a brief sonication step followed by washing, substantial dye adsorption was still observed (Fig. 4), suggesting that some capsular material remained after sonication or that dye bound to noncapsular components on the cell surface.

Dye adsorption to whole cells was 2.5-fold greater for attached bacteria than for unattached bacteria (Fig. 5). Dye adsorption to the exopolymer fraction, however, was sixfold greater for attached bacteria than for unattached bacteria (Fig. 5).

We used the alcian blue assay to find out whether the

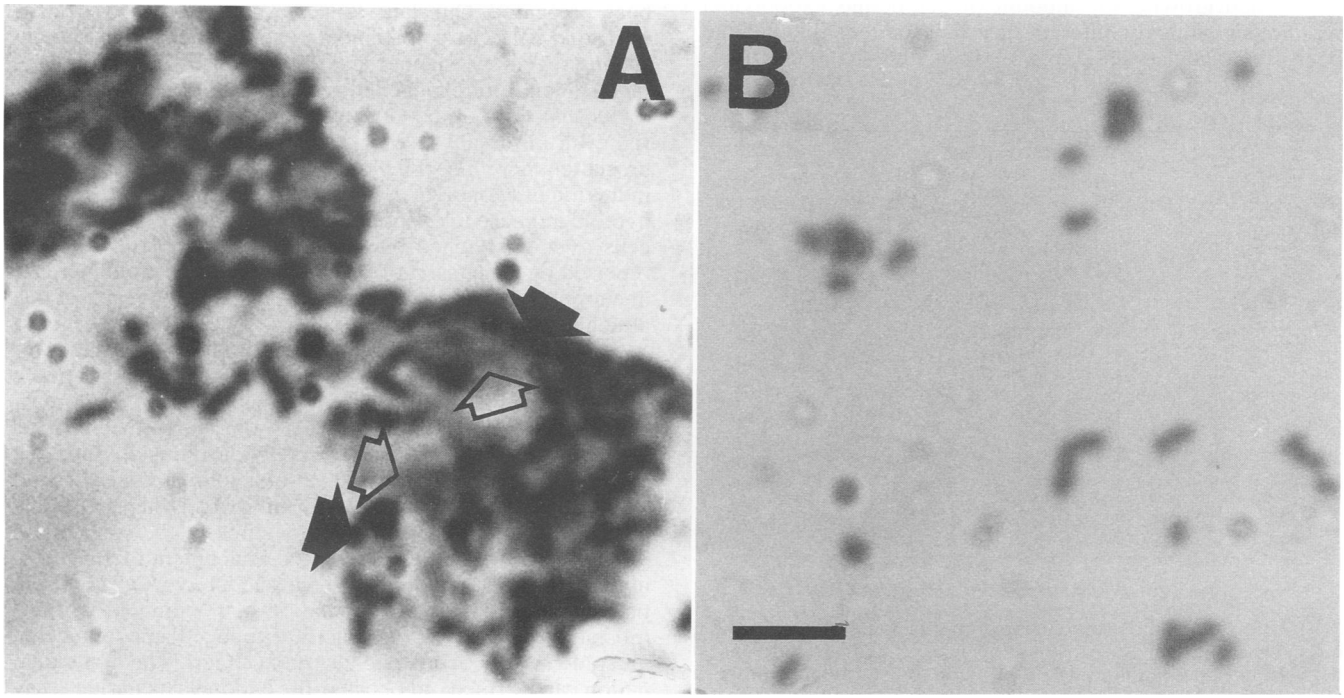

FIG. 3. (A) Microcolony extracted from a sand column after 8 days and stained with crystal violet showing bacteria (filled arrows) surrounded by exopolymers (open arrows). (B) In contrast, no exopolymer could be detected on unattached cells collected in the effluent stream. Bar, approximately 5 μm.

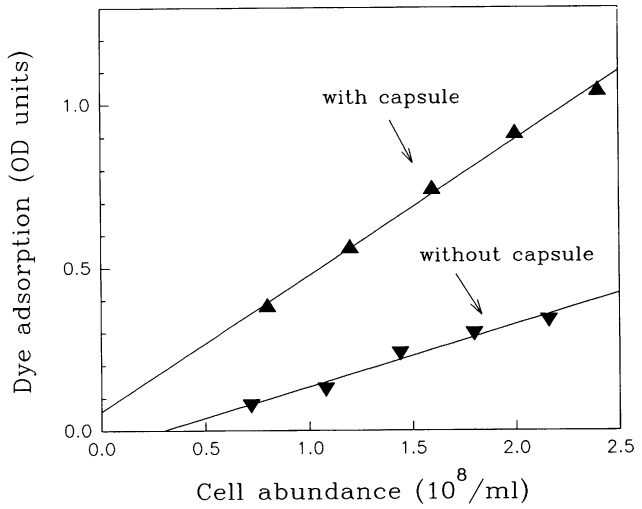

FIG. 4. Alcian blue adsorption to cells grown on a PYG plate (approximately 1-μm-thick capsules), with and without prior removal of the capsular material. OD, optical density at 606 nm.

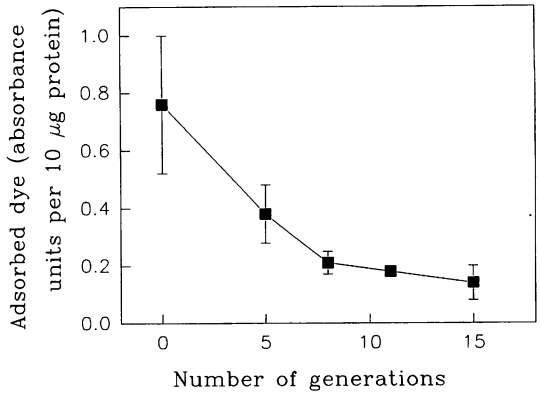

FIG. 6. Cell-bound exopolymers as a function of the number of generations after detachment. Values are for whole-cell suspensions. Growth was maintained by repeated transfers to fresh medium. Values are the means of three independent measurements (± standard deviations [error bars]), except for the first generation (n = 6).

greater amount of exopolysaccharides associated with attached cells may have resulted from the presence at the surface of a subpopulation of mucoid variants. To test for this possibility, attached cells were resuspended in a flask with fresh flow medium, i.e., the same medium used for the attached population, but not previously exposed to bacteria. Growth and polymer amounts were measured over time for five generations, as these bacteria grew unattached in the fresh medium. The amount of dye binding to cells recovered from the sand decreased markedly as they continued growing in an unattached state (Fig. 6). After five generations of unattached growth, the amount of cell-bound exopolymer was not significantly different than that for cells that had not been previously attached (Fig. 6), indicating that the surface-induced stimulation of exopolymer synthesis was reversible and, therefore, that the attached cells did not comprise a subpopulation of mucoid variants.

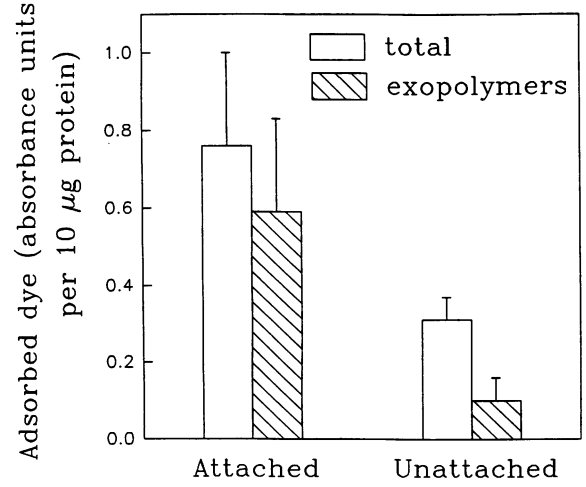

FIG. 5. Cell-bound exopolymers for attached and unattached cells. Untreated cell suspensions (open bars) and the differences between dye adsorption to whole cells and to cells stripped of their exopolymer coating (shaded bars) are shown. Attached-cell samples (n = 6) were recovered after 9 to 13 days, and unattached-cell samples (n = 9) were harvested from all stages of the growth curve. Bar, one standard deviation.

DISCUSSION

Bacterial attachment has often been described by a two-step process in which initial reversible sorption is followed by the synthesis of exopolymers anchoring the cells irreversibly (16). This model thus implies that the solid-liquid interface nearby reversibly sorbed cells triggers or somehow enhances exopolymer synthesis. Conclusive experimental evidence in support of this model has not to our knowledge been presented. Our data, however, support the hypothesis that solid surfaces can stimulate exopolysaccharide synthesis in a reversible manner and that this effect can take place independently of changes in growth conditions.

Because cells of the CAP isolate produced capsular material even in the absence of a solid-liquid interface, the data presented here describe an enhancement rather than an induction of exopolymer synthesis by attached bacteria. The fivefold increase in exopolysaccharide synthesis by attached cells over unattached ones (Fig. 2) matches well the data reported by Bengtsson (4), who observed a fivefold increase in exopolysaccharide synthesis apparently as a result of cell attachment.

However, a greater exopolysaccharide/protein ratio for attached cells does not necessarily demonstrate a surface effect on exopolysaccharide synthesis, because this observation can be explained by various alternative mechanisms such as (i) prevention of exopolymer diffusion into the surrounding fluid in the presence of a solid surface (i.e., no surface effect), (ii) detachment of exopolymer-producing cells, leaving behind exopolymers stuck to the solid phase (no surface effect), (iii) preferential attachment of a subpopulation of mucoid variants (no surface effect), (iv) preferential detachment of a subpopulation of nonmucoid variants (no surface effect), or (v) surface-induced appearance of mucoid variants (irreversible surface effect). The first explanation can be ruled out because the exopolysaccharide assay included both cell-bound and non-cell-bound exopolysaccharide. Neither is the second explanation consistent with our data, because if it were correct, the exopolysaccharide/

protein ratio in the biofilm should have increased gradually but this was not observed. The last three explanations, which assume the existence of subpopulations differing in the exopolymer synthesis (mucoid or nonmucoid variants), can also be discounted, because attached cells in our experiments did not comprise a subpopulation of mucoid variants. This conclusion is based on the finding that the synthesis of exopolymer by attached cells decreased after cell detachment to a level similar to that of cells not previously attached. Thus, it appears that only reversible surface-induced stimulation of exopolymer synthesis is consistent with our data.

What characteristic of the solid-liquid interface stimulates exopolymer synthesis by attached bacteria? According to Marshall (17), many of the physiological responses at surfaces are probably nutritional. These bacteria varied greatly in size and shape, suggesting a wide range of growth rates or stages within sessile populations that may not be well characterized by the growth rate measured for the whole population. Exopolymer synthesis may vary with specific growth rate (27) or growth stage (24, 27, 28). In fact, as the specific growth rates of isolates B0577 and B0428 were decreased approximately 10-fold in fed-batch cultures, the total carbohydrate/protein ratio increased about threefold (data not shown). For the CAP isolate, however, changes in the rate or stage of growth had little effect on exopolymer synthesis (Fig. 1B and 2B) and therefore could not account for the increased exopolymer synthesis by attached bacteria.

Exopolymer production may also change as a function of the type of limiting nutrient (19, 27). Batch cultures with flow medium were C-limited, as indicated by the fivefold increase in cell yield following a fivefold increase in citrate concentration (data not shown). Thus, the medium surrounding attached bacteria in the sand columns would have to be enriched in citrate at least fivefold compared with the bulk liquid before a nutrient other than citrate would limit growth of attached bacteria. Since this possibility seems unlikely, we conclude that the type of nutrient limitation was identical for both attached and unattached cells and therefore could not have caused the increased exopolymer synthesis at the surface.

Another limiting element for attached aerobic bacteria may be oxygen. Attached cells may encounter low oxygen tensions because respiration exceeds input of oxygen via diffusion. In our experiments, however, this seems unlikely because attached cell density was low, since we used a dilute nutrient medium (100 μM citrate).

Another possibility is that some physicochemical characteristic of the solid-liquid interface specifically activates exopolymer production without changing the nutritional status of the cells. Direct, i.e., other than nutritional, influence of solid surfaces on bacterial activity at the level of gene transcription has already been demonstrated (3, 8, 9, 21). Conceivably, surfaces also directly influence exopolymer synthesis, because membrane-bound sensory proteins affecting exopolymer synthesis have been demonstrated in bacteria belonging to several genera (6). The environmental signals to which these signal-transducing proteins may be responding have not been identified yet. Such signals may involve the presence of sorbed surfactants (15) or altered concentration and composition of electrolytes in the vicinity of a negatively charged surface such as silica sand. Alternatively, the interaction between cell surface macromolecules and the solid substratum may change the structure of the cell surface which, in turn, may elicit a physiological response via membrane sensors. Other treatments that alter the structure of the cell surface, such as desiccation or dehydration with solvents, stimulate exopolysaccharide production by *Pseudomonas* spp. (8, 20).

There may be several advantages that adherent bacteria could derive from turning on exopolymer synthesis at solid surfaces. One possible advantage is suggested by the following two observations. First, the solid-water interface has often been shown to be more favorable for growth than the surrounding bulk liquid, especially in oligotrophic conditions (reviewed in reference 26). Second, the production of exopolymers is, at least for certain bacteria (1), required to prevent desorption of daughter cells. Thus, one possible function of exopolymer synthesis at a surface is to secure individual cells and their progeny in a favorable environment. This model may explain the earlier finding (4) that exopolymer production by an attached bacterium was greater under oligotrophic conditions than following nutrient stimulation.

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